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Mechanism of D-Cycloserine Action: Alanine Racemase from *Escherichia coli* W[1]

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The antibiotic D-cycloserine is an effective inhibitor of alanine racemase. The lack of inhibition by L-cycloserine of alanine racemase from *Staphylococcus aureus* led Roze and Strominger to formulate the cycloserine hypothesis. This hypothesis states that D-cycloserine has the conformation required of the substrates on the enzyme surface and that L-cycloserine cannot have this conformation. Alanine racemase from *Escherichia coli* W has been examined to establish whether these observations are a general feature of all alanine racemases. The enzyme (molecular weight = 95,000) has Michaelis-Menten constants of 4.6×10^{-4} M and 9.7×10^{-4} M for D- and L-alanine, respectively. The ratio of V_{max} in the L- to D-direction to the V_{max} in the D- to L-direction is 2.3. The equilibrium constant calculated from the Haldane relationship is 1.11 ± 0.15. Both D- and L-cycloserine are competitive inhibitors with constants (K_i) of 6.5×10^{-4} M and 2.1×10^{-3} M, respectively. The ratio of K_m D-alanine to K_i D-cycloserine is 0.71, and the ratio of K_m L-alanine to K_i L-cycloserine is 0.46. Since L-cycloserine is an effective inhibitor, it is concluded that the cycloserine hypothesis does not apply to the enzyme from *E. coli* W.

Alanine racemase is the initial enzyme in the alanine branch of the pathway for the biosynthesis of uridine diphosphate (UDP)-*N*-acetylmuramyl-pentapeptide, a precursor of cell wall peptidoglycan (22). Two antibiotics that inhibit the formation of peptidoglycan, D-cycloserine and *O*-carbamyl-D-serine, are inhibitors of this enzyme (19, 31). D-Cycloserine is also an effective inhibitor of D-alanine:D-alanine ligase, the second enzyme in the alanine branch (24, 31). The inhibition by these antibiotics is accompanied by the accumulation of the nucleotide intermediate, UDP-*N*-acetylmuramyl-tripeptide (7, 19, 30). As a result of studies on alanine racemase from *Staphylococcus aureus*, Roze and Strominger (27) proposed the cycloserine hypothesis. The basic premise of this hypothesis is that D-cycloserine has the conformation required of the substrates, L- and D-alanine, on the enzyme surface.

Alanine racemases have been detected in many genera of bacteria (28, 29, 33), and many of these racemases have been purified and partially characterized (8, 11, 12, 14, 15, 19, 20, 26, 27, 31, 33). Since the cycloserine hypothesis is instrumental to our understanding of D-cycloserine action, it is of primary concern to establish whether the observations which led to this hypothesis are a general feature of alanine racemases. Thus, it is the purpose of this communication to examine the alanine racemase from *Escherichia coli* and establish whether the constraints of the hypothesis are consistent with the intrinsic properties of this enzyme.

MATERIALS AND METHODS

Materials. L-[*1*-^{14}C]Alanine and D-[*1*-^{14}C]alanine were purchased from New England Nuclear Corp., and purified by passage over Dowex 50 (H^+). The specific activities were 13.2 and 13.5 μCi/μmole, respectively. L-Alanine and D-alanine were purchased from Cyclo Chemical Corp. Glutamic-pyruvic transaminase (EC 2.6.1.2, 27 units/mg) was the product of the Boehringer Mannheim Corp. D-Amino acid oxidase (EC 1.4.3.3, 2.3 units/mg) and catalase (EC 1.11.1.6, 7.1×10^4 units/mg) were purchased from Worthington Biochemical Corp. Sephadex products and four of the proteins used in the calibration of the Sephadex column were obtained from Pharmacia. Hemerythrin azide was a gift from R. Rill. We thank Roger Harned of Commercial Solvents Corp., for

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generous samples of D-cycloserine and *O*-carbamyl-D-serine.

L-Cycloserine was purchased from Calbiochem, Inc. Its purity was checked by three methods: (i) optical rotation and circular dichroism at 226 and 231 nm, respectively; (ii) C H N analysis; and (iii) paper chromatography in 77% ethanol-water. D- and L-β-aminoxyalanine were synthesized in this laboratory by N. Bisgard. All other chemicals were reagent grade.

Purification of alanine racemase. (i) Growth of bacteria. *E. coli* W (ATCC 9637) was maintained in vacuo at −20 C. Bacteria were grown in lots of 13 liters in the minimal medium containing 0.1% NH_4Cl, 0.68% $NaH_2PO_4 \cdot H_2O$, 0.30% K_2HPO_4, 0.02% $MgSO_4$, 0.05% NaCl, and 0.15% L-alanine (1.7×10^{-2} M). The medium was inoculated with a 12-hr culture (500 ml), and the bacteria were grown under aerobic conditions for 13 hr to late-log phase ($\sim 8 \times 10^8$ viable cells/ml) at 37 C. The cells were harvested and washed with 20 mM tris(hydroxymethyl)-aminomethane liters (Tris)-hydrochloride, *p*H 8.0. The yield of bacteria was 20 g/13. The cells were suspended in the above buffer to make a 15% (w/v) suspension and frozen at −20 C.

(ii) Disruption of bacteria. All subsequent steps were carried out at 0 to 4 C unless otherwise stated. A portion of the 15% suspension of bacteria was disrupted in 40-ml lots by shaking for 5 min with 35 g of washed glass beads (0.11 μm, Will Corp.) in a Bronwill mechanical homogenizer (Braun model, MSK, 4,000 cycles/min). The beads were removed by filtration, and the suspension was centrifuged at 14,000 × *g* for 15 min to remove unbroken cells and cell walls.

(iii) Protamine sulfate treatment. To the turbid supernatant fraction, a 1% solution of protamine sulfate (adjusted to *p*H 5.0) was added dropwise in the proportion of 1.5 ml of protamine sulfate to 10.0 ml of cell-free extract. The solution was stirred an additional 15 min before the precipitate was removed by centrifugation at 14,000 × *g*. The supernatant fraction was adjusted to *p*H 8.0.

(iv) Butanol treatment. To the supernatant fraction was added dropwise one-half its volume of cold (0 C) 1-butanol. The two-phase solution was stirred an additional 30 min and centrifuged at 14,000 × *g* for 20 min. The aqueous phase was retained, and ammonium sulfate was added to 30% saturation to reduce the butanol in solution. After centrifugation at 14,000 × *g*, the supernatant fraction was dialyzed overnight against 20 mM Tris-hydrochloride, *p*H 8.0.

(v) Ammonium sulfate fractionation. The dialyzed supernatant fraction was brought to 40% saturation by the slow addition of solid ammonium sulfate. The solution was stirred an additional 15 min and centrifuged at 14,000 × *g* for 20 min. To the supernatant fraction, additional solid ammonium sulfate was added to increase the saturation to 60%. The precipitate was collected by centrifugation at 14,000 × *g* for 20 min, dissolved in a minimal volume of 20 mM Tris-hydrochloride, *p*H 8.0, and dialyzed overnight against 20 mM Tris-hydrochloride buffer. The dialyzed solution was stored in liquid nitrogen.

(vi) Chromatography on DEAE-Sephadex (I). A portion (~30%) of the dialyzed 40 to 60% ammonium sulfate fraction was applied to a diethylaminoethyl (DEAE)-Sephadex column (0.9 by 60 cm) previously equilibrated with 0.1 M NaCl in 20 mM Tris-hydrochloride, *p*H 8.0. The column was eluted with a linear gradient from 0.1 M to 0.4 M NaCl in 20 mM Tris-hydrochloride, *p*H 8.0. The total volume of the gradient was 200 ml. The enzyme was eluted at approximately 0.25 M NaCl. Fractions containing the major portion of the activity were combined and brought to 60% saturation with ammonium sulfate. The precipitate was collected by centrifugation and dissolved in a minimal volume of 20 mM Tris-hydrochloride, *p*H 8.0. The fraction was dialyzed overnight and stored in liquid nitrogen.

(vii) Rechromatography on DEAE-Sephadex (II). The enzyme preparation from step vi was applied to a similar DEAE-Sephadex column, previously equilibrated with 0.15 M NaCl in 20 mM Tris-hydrochloride buffer, *p*H 8.0, and was eluted with a linear gradient between 0.15 M and 0.35 M NaCl in 20 mM Tris-hydrochloride buffer, *p*H 8.0. The enzyme was eluted at 0.20 to 0.25 M NaCl. The enzyme preparation was concentrated and dialyzed in collodion bags with a glass suction apparatus (Schleicher & Schuell Co.) at a pressure of 500 mm of mercury.

The DEAE II enzyme was purified at least 100-fold compared to the cell-free extract (Table 1). On two occasions a purification of up to 200-fold has been achieved by this procedure. The enzyme that was stored in liquid nitrogen was stable for at least 3 months.

Measurement of enzymatic activity. Alanine racemase was assayed in both directions by converting the product of the reaction to pyruvate. In the forward direction (L-alanine-to-D-alanine assay), D-alanine was deaminated with D-amino acid oxidase, and in the reverse direction (D-alanine-to-L-alanine assay) L-alanine was deaminated with glutamic-pyruvic acid transaminase. The [^{14}C]pyruvate was separated from the substrate by ion-exchange chromatography.

A standard reaction mixture (first stage) contained: 50 mM sodium phosphate buffer, *p*H 8.0; 5 mM D-[^{14}C]alanine (1.8×10^5 counts per min per μmole) or 5 mM L-[^{14}C]alanine (1.8×10^6 counts per min per μmole); and enzyme in a volume of 0.100 ml. The enzyme was added to the reaction mixture at 25 C and incubated for 10 min. The reaction was terminated by placing the tube in a boiling-water bath for 2 min.

In the second stage for the determination of D-alanine in the forward reaction (L-alanine-to-D-alanine assay), 0.020 ml of a solution containing 4.6 units of D-amino acid oxidase, 27 units of catalase, 0.2 nmole of flavine adenine dinucleotide (FAD), and 0.2 μmole of sodium pyrophosphate buffer, *p*H 8.3, was added to the reaction mixture (0.1 ml, first stage) and incubated at 37 C for 1 hr. The reaction was terminated with 0.5 ml of 0.2 N sodium citrate buffer, *p*H 2.2.

For the deamination of L-alanine in the reverse

TABLE 1. *Purification of alanine racemase*

Purification step	Volume (ml)	Protein (mg)	Total units[a]	Specific activity (units/mg)	Yield (%)
Original extract	120	1,700	26,200	15.4	100
Protamine sulfate	128	1,230	25,900	21.0	99
Butanol treatment	164	426	16,600	38.9	63
$(NH_4)_2SO_4$ (40–60%)	11.0	118	16,000	136	61
DEAE Sephadex chromatography (I)	11.5	6.7	5,460	812	21
DEAE Sephadex chromatography (II)	9.0	1.2	1,870	1,670	7

[a] The L-alanine-to-D-alanine assay was routinely used to monitor enzyme activity.

reaction (D-alanine-to-L-alanine assay) 0.020 ml of a solution containing 0.22 units of glutamic-pyruvic transaminase and 32 μmoles of α-ketoglutarate adjusted to *p*H 8.0 with NaOH was added to the reaction mixture (0.1 ml, first stage) and incubated at 37 C for 45 min. The reaction was terminated with the addition of 0.1 ml of 1 N HCl and 0.4 ml of 0.2 N sodium citrate, *p*H 2.2.

The labeled pyruvic acid in both assays was separated from [^{14}C]alanine on a Dowex 50(Na^+) X-8 (200–400 mesh) column (5 by 25 mm). The reaction mixture from the second stage was quantitatively applied to the column and eluted with 0.7- and 0.8-ml portions of 0.2 N sodium citrate, *p*H 2.2. The eluate (2.1 ml) was collected in a polyethylene vial for the determination of radioactivity. Under these conditions, [^{14}C]alanine was eluted between 6 and 10 ml. Appropriate controls were performed in each series to correct for the slow decomposition of the [^{14}C]alanine to compounds not retained by the column. The amount of D-amino acid oxidase and glutamic-pyruvic acid transaminase was sufficient to deaminate 100 nmoles of D- or L-alanine. The usual amount of D- or L-alanine converted was between 5 and 50 nmoles. Not more than 10% of the alanine was converted to product.

Both assays were linear with respect to time and enzyme concentration. A unit of enzyme is defined as that amount which will catalyze the formation of 1 μmole of product in 1 hr at 25 C.

Molecular weight determination—gel filtration. A Sephadex G-150 column (0.9 by 55 cm) was equilibrated at 4 C with 20 mM Tris-hydrochloride, *p*H 8.0, at a pressure of 17 cm water. The column was developed at the same pressure at a rate of 3.4 ml/hr. Samples (1.5 mg) of ribonuclease A, chymotrypsinogen A, ovalbumin, aldolase, and hemerythrin azide were used to calibrate the column by the method of Andrews (4). A sample of enzyme (DEAE II, 52 units) was applied to the column in a volume of 1.0 ml and eluted with the above buffer. Column fractions were assayed for activity with the L-alanine-to-D-alanine assay.

Sucrose density gradient centrifugation. Sucrose gradients (5–40%) were prepared in centrifuge tubes (5 ml). Two of the reference proteins plus the racemase were layered on each gradient for calibration by the method of Martin and Ames (21). After centrifugation at 114,000 × *g* for 18 hr, the tubes were punctured and fractions of 0.2 ml were collected for determination of enzyme activity and protein.

Analytical methods. Protein was determined by the method of Lowry et al. (18). Measurements of radioactivity were made in polyethylene vials with a liquid scintillation spectrometer. The scintillation fluid was Triton X-100-toluene (1:2, v/v) described by Patterson and Green (25) and evaluated by Benson (5). Kinetic constants were determined from initial velocity measurements with a computer program which utilized a least-squares fit of points for Lineweaver-Burk (17) and Eadie (10) plots.

RESULTS

For the purification of alanine racemase, *E. coli* was grown on minimal medium containing L-alanine as the carbon source; growth on this carbon source results in a 25-fold higher level of alanine racemase when compared with growth on glucose (16). The specific activity was increased 100-fold by a combination of protamine-sulfate treatment, butanol treatment, ammonium-sulfate precipitation, and two-column chromatographic steps on DEAE-Sephadex (see above). The results of a typical purification are shown in Table 1. Samples of the enzyme preparations from each stage of purification were analyzed by electrophoresis on polyacrylamide disc gels. The enzyme of highest purity (DEAE II-enzyme, specific activity = 1,670 units/mg) showed two bands of approximately equal intensity, one of which contained alanine racemase.

Molecular weight. The molecular weight of alanine racemase was estimated by gel filtration by the method of Andrews (4) on Sephadex G-150 and by sedimentation in a sucrose density gradient by the method of Martin and Ames (21). A value of 98,000 ± 4,000 was obtained by gel filtration (Fig. 1A), and a value of 92,000 ± 5,000 was established from the sucrose density gradient analysis (Fig. 1B). The average of these values, 95,000, is to be compared with the values of 60,000 for alanine racemase from *Pseudomonas putida* (26) and 100,000 for alanine racemase from *Bacillus*

subtilis (M. P., Thornton, Ph.D. thesis, Univ. of Nebraska, 1967).

Cofactor requirement. Alanine racemase purified from either *Streptococcus faecalis* (33), *Bacillus subtilis* (8) or *Pseudomonas putida* (26) required the addition of pyridoxal phosphate for full activity. In addition, FAD has been implicated as a cofactor in the reaction catalyzed by alanine racemase from *B. subtilis*. No direct evidence for pyridoxal phosphate as a cofactor has been obtained for the enzyme from *E. coli*. However, inhibition by aminoxyacetic acid and H_2NOH (see Table 2) suggested that a functional carbonyl is required for catalytic activity.

TABLE 2. *Inhibition of alanine racemase*[a]

Inhibitor	K_i (M)	
	L → D Assay	D → L Assay
DL-1-Aminoethylphosphonic acid[b]	1.8×10^{-5}	—[c]
Aminoxyacetic acid	4.5×10^{-8}	—[c]
β-Aminoxy-D-alanine	—[d]	2.5×10^{-5}
β-Aminoxy-L-alanine	—[c]	1.6×10^{-4}
O-Carbamyl-D-serine[e]	7.4×10^{-4}	7.1×10^{-4}
D-Cycloserine[f]	6.5×10^{-4}	6.8×10^{-4}
L-Cycloserine[g]	2.1×10^{-3}	—[h]
Hydroxylamine	4.7×10^{-5}	—[c]
cis-DL-Cyclothreonine	$\sim 1.2 \times 10^{-2}$	—[c]

[a] The following compounds had a $K_i > 2 \times 10^{-2}$ M: *trans*-DL-cyclothreonine, aminoisobutyric acid, DL-2-aminoethylphosphonic acid, β-alanine, L-α-amino-*n*-butyric acid, D-α-amino-*n*-butyric acid, L-serine, D-serine, L-norvaline, L-threonine, D-threonine, and D-cysteine.

[b] From Fig. 6.

[c] Not tested.

[d] β-Aminoxy-D-alanine is an effective inhibitor of D-amino acid oxidase.

[e] From Fig. 7C and D.

[f] From Fig. 7A and B.

[g] From Fig. 5.

[h] L-Cycloserine is an effective inhibitor of the glutamic-pyruvic transaminase.

pH Optima. The *p*H optima of the reaction in the L → D direction and the reaction in the D → L direction are in the range from 9 to 10 (Fig. 2A). The ratio of the activities in the two directions significantly decreases with increasing values of *p*H (Fig. 2B). For example, at *p*H 8.0 the ratio is 4.8 and at *p*H 9.5 the ratio is 2.1. Another characteristic of these data is the difference in *p*H required for 50% of the maximal activity. In the L → D direction, the *p*H for 50% of the activity on the acid side is 7.6, whereas in the D → L direction the *p*H for 50% of the activity is 8.5.

Kinetic parameters. A kinetic analysis of alanine racemase indicated significant differences in the maximal velocities and Michaelis-Menten constants of the substrates in the forward (L → D) and reverse (D → L) directions. The constants were established from the Lineweaver-Burk plots (17) presented in Fig. 3A and B. From these data, the values for K_m of D-and L-alanine are $(4.6 \pm 0.1) \times 10^{-4}$ M and $(9.7 \pm 0.4) \times 10^{-4}$ M, respectively. From Eadie plots (10), almost identical values to those cal-

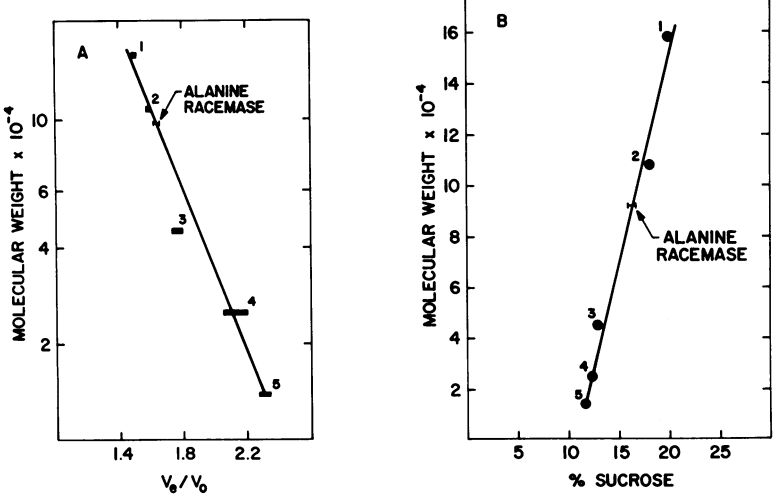

FIG. 1. *Estimation of the molecular weight of alanine racemase by gel filtration on Sephadex G-150 (A) and by sedimentation in a sucrose density gradient (B). In A and B the standards are: (1) aldolase, 158,000; (2) hemerythrin azide, 108,000; (3) ovalbumin, 45,000; (4) chymotrypsinogen A, 25,000; and (5) ribonuclease A, 13,700. V_e/V_0 is the ratio of the elution volume of the sample compared to the void volume determined for blue dextran. V_e/V_0 for alanine racemase is 1.64.*

TABLE 3. *Summary of inhibitor constants for alanine racemase*

Source	K_i (M)	
	D-Cycloserine (moles/liter)	L-Cycloserine (moles/liter)
Streptococcus faecalis[a]	2.4×10^{-4}	>>0.01
Staphylococcus aureus[b]	5.0×10^{-5}	>>0.01
Escherichia coli	6.5×10^{-4}	2.1×10^{-3}
Bacillus subtilis[c]	$\sim 1.0 \times 10^{-3}$	$\sim 5.0 \times 10^{-4}$

[a] Lynch and Neuhaus (19), Neuhaus (23).
[b] Roze and Strominger (27).
[c] Johnston et al. (15).

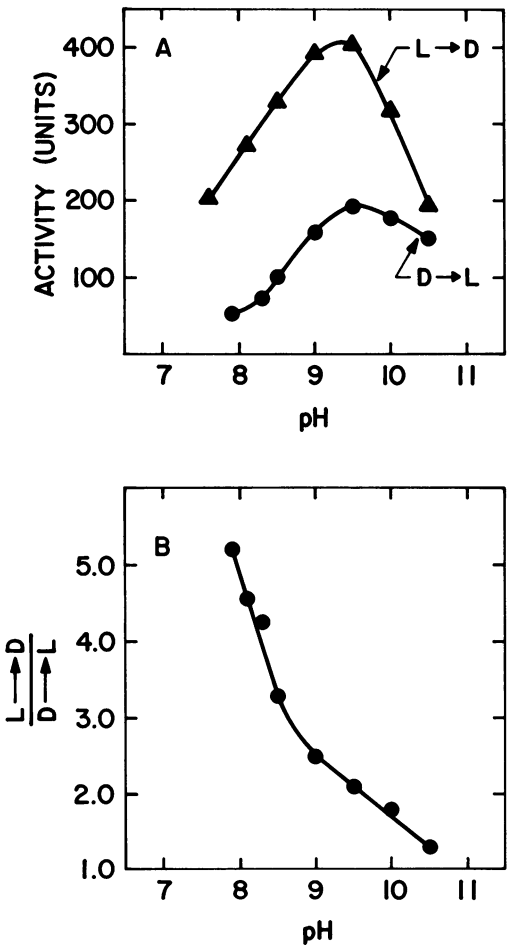

FIG. 2. *pH Optima. In A, the L-alanine-to-D-alanine and the D-alanine-to-L-alanine assays were used. The reactions were initiated by adding enzyme (50 ng, DEAE II) to the assay mixture at the indicated pH. The combination buffer contained 100 mM Na_2CO_3, 100 mM H_3BO_4, 100 mM NaCl, and 100 mM Na_2HPO_4 and was adjusted to the indicated pH with NaOH or HCl. In B, the ratio of the L-alanine-to-D-alanine assay to the D-alanine-to-L-alanine assay is presented.*

culated from Lineweaver-Burk plots were obtained. The values of V_{max} in the L → D and D → L direction are 2.22 ± 0.08 and 0.95 ± 0.04 μmoles/hr, respectively. Since the K_{eq} for this reaction is 1, the validity of these constants can be examined in the Haldane relationship:

$$K_{eq} = \frac{(K_m \text{ D-alanine})(V_{max} \text{ L} \rightarrow \text{D})}{(K_m \text{ L-alanine})(V_{max} \text{ D} \rightarrow \text{L})} \quad (1)$$

The calculated value for K_{eq} is 1.11 ± 0.15. One feature of the Lineweaver-Burk plot should be emphasized. As illustrated in Fig. 3B, the double reciprocal plot shows a pronounced substrate inhibition in the D-alanine-to-L-alanine assay.

Time course. The reaction in the L-alanine-to-D-alanine direction proceeds at a faster rate than in the D-alanine-to-L-alanine direction. This is also illustrated in the progress curve shown in Fig. 4. The time course can be predicted from the Michaelis-Menten constants and maximal velocities (2).

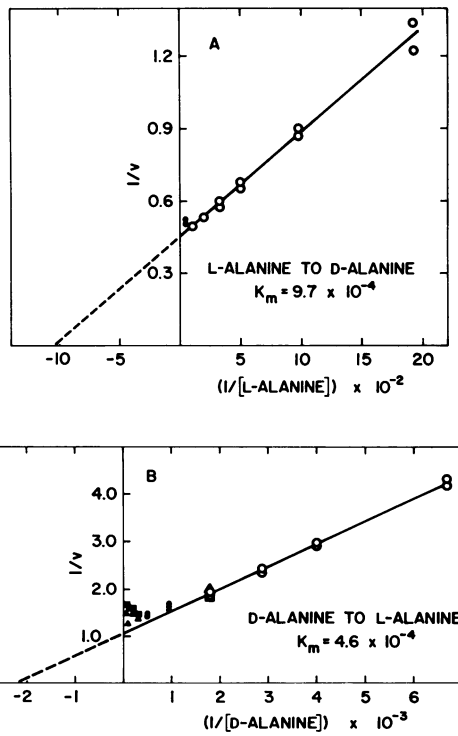

FIG. 3. *Effect of alanine on the forward (A) and reverse (B) reactions. In A the L-alanine-to-D-alanine assay was used with 50 ng of enzyme (DEAE II) and in B the D-alanine-to-L-alanine assay was used with 50 ng of enzyme (DEAE II). The results are presented as Lineweaver-Burk plots calculated from a least-squares analysis of the open points (see Materials and Methods).*

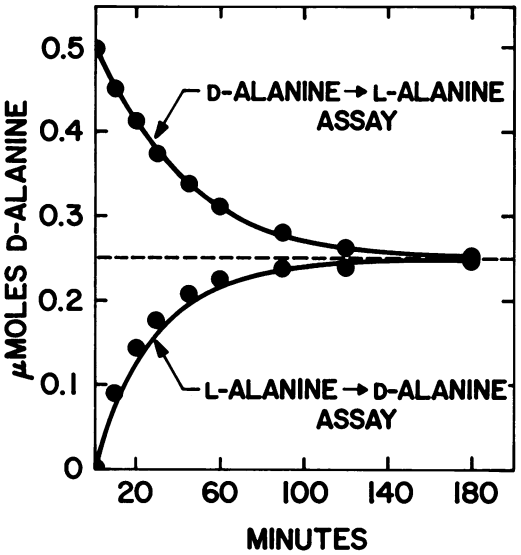

FIG. 4. *Time course of alanine racemase. In the* D-*alanine-to-*L-*alanine assay, the reaction mixture contained: 5 mM* D-*alanine (157 nCi/μmole); 50 mM phosphate buffer, pH 8.0, and 750 ng of racemase (DEAE II) per 100 μliter. In the* L-*alanine-to-*D-*alanine assay the reaction mixture contained: 5 mM* L-*alanine (144 nCi/μmole); 50 mM phosphate buffer, pH 8.0; and 750 ng of racemase (DEAE II) per 100 μliters. Samples were removed at the indicated time, and the amount of* D-*alanine was determined by the method described in the Materials and Methods. The continuous line is calculated from equation 4.*

For the equilibria

$$E + S \rightleftarrows X \rightleftarrows E + P \qquad (2)$$

the velocity can be calculated from:

$$-\frac{dS}{dt} = \frac{dP}{dt} = \frac{(V_s/K_s)S - (V_p/K_p)P}{1 + S/K_s + P/K_p} \qquad (3)$$

where S = substrate concentration and P = product concentration. Integration of equation 3 yields:

$$\frac{V_s}{K_s}\left(1 + \frac{1}{K_{eq}}\right)t = \left(\frac{1}{K_s} - \frac{1}{K_p}\right)P - \left[1 + \frac{S_o}{K_p} + \frac{(1/K_s) - (1/K_p)}{1 + K_{eq}} S_o\right] \ln\left[1 - \frac{P}{P_{eq}}\right] \qquad (4)$$

From this equation and the Michaelis-Menten constants, the continuous curves in Fig. 4 were calculated. Good agreement is observed between the experimental points and the calculated lines.

Inhibition of alanine racemase. To define the active center of the enzyme, a series of alanine analogues were tested for their action on the racemase. As illustrated in Table 2, DL-1-aminoethylphosphonic acid, aminoxyacetic acid, β-aminoxy-D-alanine, and hydroxylamine are very effective inhibitors. In addition, *O*-carbamyl-D-serine, β-aminoxy-L-alanine, D-cycloserine, and L-cycloserine are effective inhibitors.

One of the features of the inhibitor specificity profile is the significant inhibition by L-cycloserine. With alanine racemase from either *S. aureus* Copenhagen (27) or *S. faecalis* (19), no significant inhibition was observed with L-cycloserine. However, in the case of the enzyme from *E. coli*, L-cycloserine is a competitive inhibitor with a K_i of 2.1×10^{-3} M (Fig. 5). For comparison, the ratio K_m D-alanine to K_i D-cycloserine is 0.71, and the ratio K_m L-alanine to K_i L-cycloserine is 0.46.

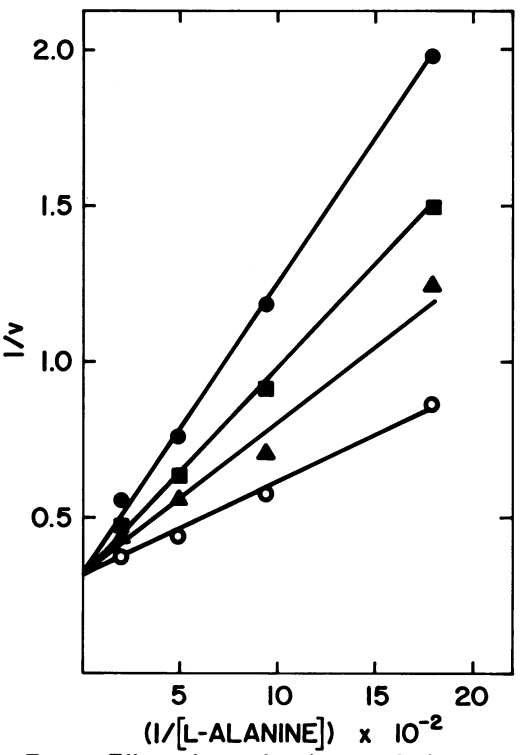

FIG. 5. *Effect of* L-*cycloserine on alanine racemase. The* L-*alanine-to-*D-*alanine assay was used with 50 ng of racemase (DEAE II). The concentrations of* L-*cycloserine are:* O, *none;* ▲, *1.5 mM;* ■, *2.5 mM; and* ●, *4 mM. The results are presented as Lineweaver-Burk plots calculated from a least-squares analysis.*

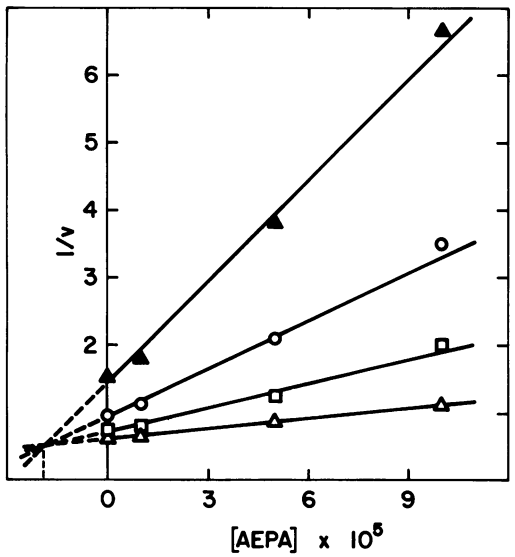

FIG. 6. *Effect of* DL-*1-aminoethylphosphonic acid (AEPA) on alanine racemase (Dixon plot). The* L-*alanine-to-*D-*alanine assay was used with 93 ng of racemase (DEAE II). The concentrations of* L-*alanine are:* △, *5 mM;* □, *2 mM;* ○, *1 mM;* ▲, *0.5 mM.*

A second feature of the inhibitor specificity profile is the effective inhibition of the racemase by DL-1-aminoethylphosphonic acid. Dulaney (9) observed that this analogue of D-alanine is an inhibitor of cell wall biosynthesis and that its action may be reversed by D-alanine. As illustrated in Fig. 6, this analogue is a competitive inhibitor of the alanine racemase from *E. coli* with a K_i of 1.8×10^{-5} M.

The inhibition of glutamic-pyruvic transaminase (13), glutamic-oxaloacetic transaminase (32), and alanine racemase (11, 27) by hydroxylamine and substituted hydroxylamines has been used to implicate pyridoxal phosphate in the action of these enzymes. With alanine racemase from *E. coli*, a significant inhibition by aminoxyacetic acid, β-aminoxy-D- and β-aminoxy-L-alanine, and hydroxylamine is observed. The inhibition by each of these compounds is competitive with alanine. In the case of aminoxyacetic acid, the inhibition is more effective ($K_i = 4.5 \times 10^{-8}$ M) than that observed for alanine racemase from *Pseudomonas* species 3550 ($K_i = 4 \times 10^{-7}$ M) (11). In addition, both β-aminoxy-D-alanine and β-aminoxy-L-alanine are more effective inhibitors of the racemase from *E. coli* than of the racemase from *S. faecalis* (23). Although the inhibition by the above carbonyl inhibitors would seem to implicate pyridoxal phosphate as a cofactor, no direct evidence has been obtained for its participation.

In an analysis of the alanine racemase from *Lactobacillus fermenti*, Johnston and Diven (14) concluded that this enzyme existed in two enzyme forms, one form that binds L-alanine and one that binds D-alanine. This conclusion was based on a detailed kinetic analysis of the inhibition by D-cysteine with both D- and L-alanine as substrates. With L-alanine as the substrate, only the slopes of the Lineweaver-Burk plots were increased. With D-alanine as substrate, both the slopes and $1/v$ intercepts were altered.

Since D-cysteine is not an effective inhibitor of alanine racemase from *E. coli*, two inhibitors, *O*-carbamyl-D-serine and D-cycloserine, were used in place of this compound to analyze the reaction kinetics. The results in Fig. 7 A, B, C, D show the action by these analogues in both the forward and reverse direction. The inhibition by both compounds is competitive with either L- or D-alanine. No change in the $1/v$ intercepts with either D- or L-alanine was observed. Thus, our results with these inhibitors are clearly different from the observation by Johnston and Diven (14) with D-cysteine on the enzyme from *L. fermenti*.

Specific effectors. Significant stimulation of alanine racemase by several compounds has been reported by other workers. For example, acetate anion enhances the activity of the enzyme from *L. fermenti* (14), and FAD stimulates the enzyme from *B. subtilis* (8). With threonine racemase from *E. coli*, Amos (3) observed that adenosine triphosphate (ATP) stimulates the racemization of this amino acid. In the case of alanine racemase from *B. subtilis*, ethylenediaminetetraacetic acid (EDTA) stabilized the enzyme during purification on carboxymethyl Sephadex (8). With alanine racemase from *L. fermenti*, both glutathione and β-mercaptoethanol increased the activity. In our experiments with alanine racemase from *E. coli*, the following compounds were without effect at 10^{-3} M: sodium acetate, ATP, FAD, EDTA, glutathione, and β-mercaptoethanol.

DISCUSSION

A kinetic and specificity analysis has defined some of the intrinsic properties of alanine racemase from *E. coli*. These analyses will contribute to an understanding of the function and regulation of this enzyme. In addition, comparative studies of the inhibition by cycloserine will test the generality of the cycloserine hypothesis proposed by Roze and Strominger (27) for the racemase from *S. au-*

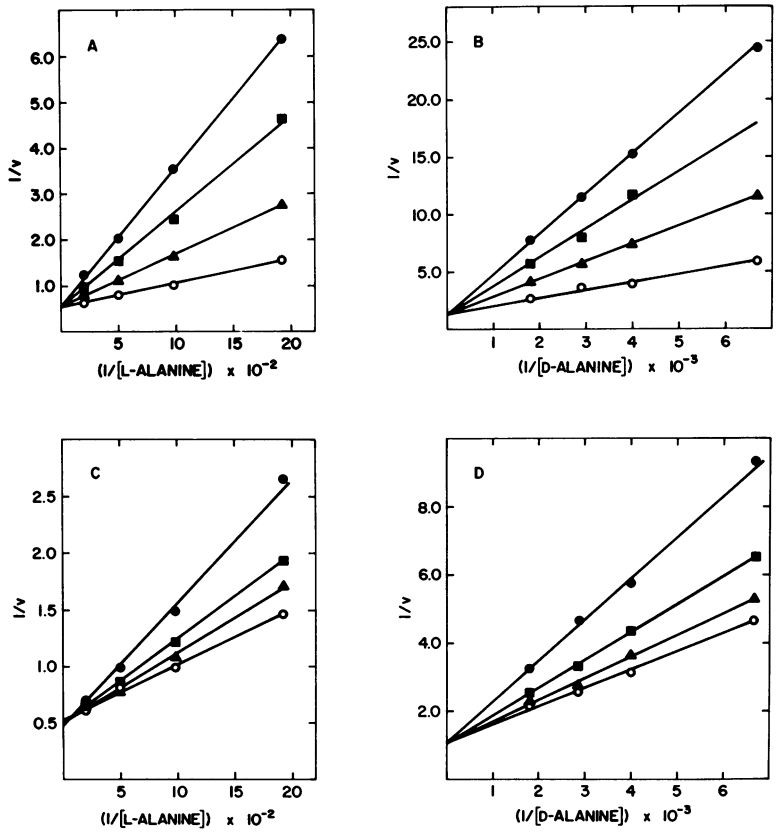

FIG. 7. *Inhibition of alanine racemase by* D-*cycloserine* (*A,B*) *and O-carbamyl-*D-*serine* (*C,D*). *The* L-*alanine-to-*D-*alanine assay* (*A,C*) *and the* D-*alanine-to-*L-*alanine assay* (*B,D*) *were used with 50 ng of enzyme* (*DEAE II*). *In A and B, the concentrations of* D-*cycloserine are:* ○, *none;* ▲, *1 mM;* ■, *2 mM;* ○, *3 mM. In C and D, the concentrations of O-carbamyl-*D-*serine are:* ○, *none;* ▲, *0.2 mM;* ■, *0.5 mM;* ●, *1 mM. The results are presented as Lineweaver-Burk plots calculated from a least-squares analysis.*

reus. Specifically, the following points of this hypothesis will be considered: (i) inhibition by L-cycloserine; (ii) interpretation of the ratio K_m/K_i; (iii) evidence for a single alanine binding site.

From Lineweaver-Burk plots, the Michaelis-Menten constants were determined for D- and L-alanine. The K_m for D-alanine is 4.6×10^{-4} M, and the K_m for L-alanine is 9.7×10^{-4} M. These values are lower than those reported for the enzyme from *S. faecalis* R [6.8×10^{-3} M for L-alanine, 3.0×10^{-3} M for D-alanine (19), 8.5×10^{-3} M for L-alanine (33)]; *S. aureus* (4×10^{-3} to 6×10^{-3} M for L- and D-alanine) (27); *Pseudomonas* species 3550 (3×10^{-2} M for L-alanine) (11); *P. putida* (3×10^{-2} M for L-alanine) (26); and *L. fermenti* (10^{-2} M for L-alanine, 7.3×10^{-3} M for D-alanine) (14). Thus, among those racemases that have been examined, the enzyme from *E. coli* requires a significantly lower concentration of alanine for one-half maximal velocity than the other racemases. As in the case of alanine racemase from *S. faecalis* (19), the Michaelis-Menten constants for the L- and D-isomers are significantly different. Similar observations have been made with hydroxyproline 2-epimerase (1) and proline racemase (6). The maximal velocity in the L-alanine to D-alanine direction is twofold larger than the maximal velocity in the D-alanine to L-alanine direction. This difference has also been noted with the partially purified racemase from *S. faecalis* (19). The validity of the Michaelis-Menten constants and V_{max} values has been assessed in the Haldane relationship and the integrated form of the rate equation. In the latter case, the time course of the reaction is in good agreement with that calculated from the rate equation that is based on experimental values of K_m and V_{max}.

In studies on alanine racemase, D-cycloserine, L-cycloserine, and *O*-carbamyl-D-serine

have been instrumental in probing the reaction catalyzed by the enzyme (19, 23, 27). As a result of work on alanine racemase from *S. aureus*, Roze and Strominger (27) formulated the cycloserine hypothesis. This hypothesis is based on the proposal that D- and L-alanine, when bound to the enzyme, have the same conformation with respect to the functional groups ($-NH_3^+$ and $-COO^-$) and that this conformation is that found in D-cycloserine. The proposal is based on the lack of inhibition by L-cycloserine observed with the alanine racemase prepared from *S. aureus*. As shown in Table 3, the inhibition by L-cycloserine is a function of the enzyme source. For example, L-cycloserine inhibits both the enzyme from *E. coli* and that from *B. subtilis* whereas it does not significantly inhibit the racemase from *S. aureus* and *S. faecalis*.

In analyzing the data for the alanine racemase from *S. aureus*, Roze and Strominger (27) observed that the cycloserine hypothesis provides an explanation of the fact that the K_i for the inhibitor (D-cycloserine) is 100 times smaller than the K_m for D- or L-alanine. They proposed that the antibiotic is fixed in the conformation preferred by the enzyme, in contrast to the substrates which have many possible conformations. Consequently, at the same concentration, the number of molecules of D-cycloserine in the conformation required by the enzyme is much greater than the number of molecules of either of the substrates in the corresponding conformation. In the case of the alanine racemase from *S. faecalis*, the ratio of K_m of D-alanine to K_i of D-cycloserine is 12.8, and with the enzyme from *E. coli* the ratio of K_m of D-alanine to K_i of D-cycloserine is 0.71. Clearly, the wide variation in the ratio (K_m D-alanine to K_i D-cycloserine, 100 to 0.7) indicates that the interpretation of this ratio must be evaluated.

In explaining the lack of inhibition of alanine racemase by L-cycloserine, Roze and Strominger (27) postulated a single active site on alanine racemase, in which L- and D-alanine bind in the same conformation. If D-alanine and L-alanine bind to the same site on the enzyme, one might visualize subsites for the methyl groups in both the D- and L-configurations. If this were the case, α-amino-isobutyric acid would be expected to inhibit the racemase. No significant inhibition by this amino acid of the racemase from *E. coli* has been detected. The lack of inhibition by α-amino-isobutyric acid and the inhibition by both D- and L-cycloserine argue for two binding sites, one for D-alanine and one for L-alanine. Although our kinetic studies did not distinguish between a one-site and two-site model, we feel that the differences in Michaelis-Menten constants and maximal velocities for the L- and D-isomers are consistent with separate binding sites. In addition, from an analysis of the *p*H optima (Fig. 2B), the ratio of the velocity in the L $\rightarrow$ D direction to the velocity in the D $\rightarrow$ L direction decreases as the *p*H is increased. This change is more consistent with separate binding sites for L- and D-alanine. One feature of the Lineweaver-Burk plot should be emphasized. As illustrated in Fig. 3B, the double-reciprocal plot shows a pronounced substrate inhibition in the D- to L-alanine assay. This type of inhibition can be visualized if D-alanine binds at high substrate concentrations to the L-alanine site as well as the D-alanine site. This observation is not consistent with a single binding site for both D- and L-alanine.

In analyzing alanine racemase from *L. fermenti*, Johnston and Diven (14) obtained evidence for a model that contained two enzyme forms, one form that binds L-alanine and one form that binds D-alanine. These results were based on the inhibition of the enzyme by D-cysteine. Since D-cysteine was not an effective inhibitor of the enzyme from *E. coli*, we used D-cycloserine and *O*-carbamyl-D-serine in place of D-cysteine. With these inhibitors no change in the $1/v$ intercepts with either D- or L-alanine was observed. These results are consistent with either a one- or two-site model and are not consistent with the two-enzyme-form model.

The differences in kinetic parameters and inhibitor specificity profiles of alanine racemases from various sources suggest that the generality of the cycloserine hypothesis requires evaluation. It would appear that this hypothesis applies to the enzyme from *S. aureus* and *S. faecalis*. The racemases from *E. coli* and *B. subtilis*, however, do not appear to conform to the constraints described in the cycloserine hypothesis.

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LITERATURE CITED

1. Adams, E., and I. L. Norton. 1964. Purification and properties of inducible hydroxyproline 2-epimerase from *Pseudomonas*. J. Biol. Chem. **239**:1525–1535.

2. Alberty, R. A. 1959. Mechanisms of enzyme action. Rev. Mod. Phys. **31**:177–184.
3. Amos, H. 1954. A racemase for threonine in *Escherichia coli*. J. Amer. Chem. Soc. **76**:3858.
4. Andrews, P. 1965. The gel-filtration behavior of proteins related to their molecular weights over a wide range. Biochem. J. **96**:595–606.
5. Benson, R. H. 1966. Limitations of tritium measurements by liquid scintillation counting of emulsions. Anal. Chem. **38**:1353–1356.
6. Cardinale, G. J., and R. H. Abeles. 1968. Purification and mechanism of action of proline racemase. Biochemistry **7**:3970–3978.
7. Ciak, J., and F. E. Hahn. 1959. Mechanisms of action of antibiotics. II. Studies on the modes of action of cycloserine and its L-stereoisomer. Antibiot. Chemother. **9**:47–53.
8. Diven, W. F., J. J. Schulz, and R. B. Johnston. 1964. Purification and properties of the alanine racemase from *Bacillus subtilis*. Biochim. Biophys. Acta **85**:322–332.
9. Dulaney, E. L. 1970. 1-Aminoethylphosphonic acid, an inhibitor of bacterial cell wall synthesis. J. Antibiot. **23**:567–568.
10. Eadie, G. S. 1942. The inhibition of cholinesterase by physostigmine and prostigmine. J. Biol. Chem. **146**:85–93.
11. Free, C. A., M. Julius, P. Arnow, and G. T. Barry. 1967. Inhibition of alanine racemase by aminoxyacetic acid. Biochim. Biophys. Acta **146**:608–610.
12. Hamada, T. 1960. Isolation, purification, and properties of alanine racemase of *Bacillus brevis*. Osaka Shiritsu Daigaku Igabu Zashi **9**:1927–1938.
13. Hopper, S., and H. L. Segal. 1962. Kinetic studies of rat liver glutamic-alanine transaminase. J. Biol. Chem. **237**:3189–3195.
14. Johnston, M. M., and W. F. Diven. 1969. Studies on amino acid racemases. I. Partial purification and properties of the alanine racemase from *Lactobacillus fermenti*. J. Biol. Chem. **244**:5414–5420.
15. Johnston, R. B., J. J. Scholz, W. F. Diven, and S. Shepherd. 1968. Some further studies on the purification and mechanism of the action of alanine racemase from *Bacillus subtilis*, p. 537. *In* E. Snell, A. E. Braunstein, E. S. Severin, and Y. M. Torchinsky (ed.), Pyridoxal catalysis: enzymes and model systems. John Wiley & Sons, Inc., New York.
16. Lambert, M. P., and F. C. Neuhaus. 1972. Factors affecting the level of alanine racemase in *Escherichia coli*. J. Bacteriol. **109**:1156–1161.
17. Lineweaver, H., and D. Burk. 1934. The determination of enzyme dissociation constants. J. Amer. Chem. Soc. **56**:658–666.
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
19. Lynch, J. L., and F. C. Neuhaus. 1966. On the mechanism of action of the antibiotic *O*-carbamyl-D-serine in *Streptococcus faecalis*. J. Bacteriol. **91**:449–460.
20. Marr, A. G., and P. W. Wilson. 1954. The alanine racemase of *Brucella abortus*. Arch. Biochem. Biophys. **49**:424–433.
21. Martin, R. G., and B. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: Application to protein mixtures. J. Biol. Chem. **236**: 1372–1379.
22. Neuhaus, F. C. 1967. The antibiotics D-cycloserine and *O*-carbamyl-D-serine, p. 40–83. *In* D. Gottlieb and P. L. Shaw (ed.), Antibiotics: mechanism of action, vol. 1. Springer-Verlag, Heidelberg.
23. Neuhaus, F. C. 1968. Selective inhibition of enzymes utilizing alanine in the biosynthesis of peptidoglycan. Antimicrob. Ag. Chemother. 1967, p. 304–313.
24. Neuhaus, F. C., and J. L. Lynch. 1964. The enzymatic synthesis of D-alanyl-D-alanine. III. On the inhibition of D-alanyl-D-alanine synthetase by the antibiotic D-cycloserine. Biochemistry **3**:471–480.
25. Patterson, M. S., and R. C. Greene. 1965. Measurement of low energy beta-emitters in aqueous solution by liquid scintillation counting of emulsions. Anal. Chem. **37**:854–857.
26. Rosso, G., K. Takashima, and E. Adams. 1969. Coenzyme content of purified alanine racemase from *Pseudomonas*. Biochem. Biophys. Res. Commun. **34**:134–140.
27. Roze, U., and J. L. Strominger. 1966. Alanine racemase from *Staphylococcus aureus*: conformation of its substrates and its inhibitor, D-cycloserine. Mol. Pharmacol. **2**:92–94.
28. Soda, K., T. Osumi, T. Yorifuji, and K. Ogata. 1969. Enzymatic racemization of leucine and α-aminobutyrate. Distribution of amino acid racemase activity, identification of the organism and new assay method of enzyme. Agr. Biol. Chem. **33**:424–429.
29. Stewart, B. T., and H. O. Halvorson. 1953. Studies on the spores of aerobic bacteria. I. The occurrence of alanine racemase. J. Bacteriol. **65**:160–166.
30. Strominger, J. L., R. H. Threnn, and S. S. Scott. 1959. Oxamycin, a competitive antagonist of the incorporation of D-alanine into a uridine nucleotide in *Staphylococcus aureus*. J. Amer. Chem. Soc. **81**:3803.
31. Strominger, J. L., E. Ito, and R. H. Threnn. 1960. Competitive inhibition of enzymatic reactions by oxamycin. J. Amer. Chem. Soc. **82**:998–999.
32. Velick, S. F., and J. Vavra. 1962. A kinetic and equilibrium analysis of the glutamic oxaloacetic transaminase mechanism. J. Biol. Chem. **237**:2109–2122.
33. Wood, W. A., and I. C. Gunsalus. 1951. D-Alanine formation: a racemase in *Streptococcus faecalis*. J. Biol. Chem. **190**:403–416.